

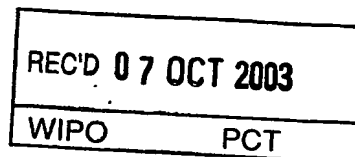


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Use of granzyme B as an Hsp70/Hsp70 peptide dependent inducer of apoptosis in
tumor cells

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Use of granzyme B as an Hsp70/Hsp70 peptide dependent inducer of apoptosis in tumor cells

10 The present invention relates to a method of inducing or enhancing the expression
of granzyme B in natural killer (NK) cells. The present invention relates also to a
use of said NK cells for the preparation of a pharmaceutical composition for the
treatment of tumors.

15 A variety of documents is cited throughout this specification. The disclosure
content of said documents is herewith incorporated by reference.

Elevated cytoplasmic levels of heat shock protein 70 (Hsp70) have been found to
protect tumor cells against programmed cell death (Nylandsted et. al. (2000) Ann.
N.Y. Acad. Sci. 926, 122). Hsp70 is the major stress inducible form of the heat
20 shock protein family (HSP), which is primarily located in the cytosol. Evidence
accumulated during recent years has demonstrated that extracellular localized and
plasma membrane-bound HSPs are highly immunogenic and expose the cells to
immune attack (Schild et. al. (1999) Current Opinion in Immunology 11, 109).
Following receptor-mediated uptake (Arnold-Schild et. al. (1999) J. Immunol. 162,
25 3757) and re-presentation by antigen presenting cells (APC), HSP-chaperoned
peptides elicit a cytotoxic, CD8⁺ T cell response (Suto et. al. (1995) *Science* 269,
1585). Several receptors, including CD91 and toll-like receptors 2 and 4 (TLR2/4),
have been identified that mediate interaction of HSP90 (gp96), HSP70 (Hsp70,
Hsc70) and HSP60 peptide complexes with APCs (Basu et. al. (2001) *Immunity*
30 14, 303; Binder et. al. (2000) *Nat. Immunol.* 1, 151; Sondermann et. al. (2000) *Biol.*

Chem. 381, 1165.; Ohashi et. al. (2000) J. Immunol. 164, 558). A peptide-independent "chaperokine-effect" has been described for members of the HSP70 group. Binding of exogenous HSP70 to monocytes via TLR2/4 in a CD14 dependent pathway induces receptor clustering and the secretion of proinflammatory cytokines via MyD88/IRAK/NF κ -B signal transduction (Pfeiffer et. al. (2001) Eur. J. Immunol. 31, 3153; Asea et. al. (2000) Nature Medicine, 6, 435; Asea et. al. (2000) Cell Stress & Chaperones, 5, 425; Asea et. al. (2002) J Biol Chem. 277(17), 15028).

- 10 Natural killer (NK) cells have been found to specifically interact with a C-terminal localized epitope of Hsp70 (Botzler et. al. (1998) Cell Stress & Chaperones, 3, 6), that is presented on the cell membrane of tumor cells (Multhoff et. al. (1995) Int. J. Cancer, 61, 272; Multhoff et. al. (1997) J. Immunol. 158, 4341). The amount of membrane-bound Hsp70 on tumor cells positively correlates with the sensitivity to
- 15 the lysis mediated by NK cells: Physical (heat) as well as chemical (cytostatic drugs) stress has been found to increase Hsp70 cell surface expression on tumor cells and thereby rendering them better targets for NK cells (Multhoff (1997) *Int. J. Hyperthermia* 13, 39; Botzler et. al. (1999) *Exp. Hematol.* 27, 470; Rabinovich et. al. (2000) J. Immunol. 165, 2390; Feng et. al. (2001) *Blood* 97, 3505). Incubation of
- 20 purified NK cells with recombinant Hsp70-protein increases their cytolytic activity against Hsp70 membrane-positive tumor cells (Multhoff et. al. (1999) *Exp. Hematology* 27, 1627). The same effect is achieved by a 14 amino acid peptide, termed TKD (TKDNNLLGRFELSG, aa450-463), derived from the C-terminal domain of Hsp70. This region corresponds to the domain of Hsp70 exposed to the
- 25 extracellular milieu of viable tumor cells (Multhoff et. al. (2001) *Cell Stress & Chaperones* 6, 337). Concomitant with an increased cytolytic activity, following contact either with Hsp70-protein or with Hsp70-peptide TKD the cell surface expression of the activating form of the C-type lectin receptor CD94 was enhanced in NK cells. Blocking assays using an inhibitory antibody specific for CD94
- 30 revealed an involvement of CD94 in the interaction of NK cells with Hsp70 membrane-positive tumor cells (Multhoff et. al. (1999) *Exp. Hematology* 27, 1627).

These data indicate that apart from HLA-E presenting leader peptides of classical HLA-alleles (Lanier et. al. (1998) Immunity 8, 693; Braud et. al. (1998) Nature 391, 795), the C-terminal localized Hsp70-peptide sequence TKD might be considered as a potential ligand for a yet undefined activating CD94 receptor complex.

- 5 Although the preceding observations indicate that Hsp70-peptide functions as a tumor-selective target recognition structure for CD94 positive NK cells (Multhoff et. al. (1997) J. Immunol. 158, 4341), the mechanism by which NK cells lyse Hsp70 positive tumor target cells remained to be elucidated. In addition, it is desirable to specifically trigger the lytic activity of NK cells towards tumor cells in a more
- 10 specific manner than has hitherto been possible. All these scientific goals serve as a means to derive more efficacious and more specific approaches to disease treatment and in particular to tumor treatment.

- Thus, the technical problem underlying the present invention was to provide means
- 15 and methods for a specific treatment of diseases and in particular of tumors.

The solution to said technical problem is achieved by providing the embodiments characterized in the claims.

- 20 Accordingly, the present invention relates to a method of inducing or enhancing the expression of granzyme B in natural killer (NK) cells comprising contacting NK cells with

- (a) Hsp70 protein;
- (b) a (C-terminal) fragment of (a) comprising the amino acid sequence
- 25 TKDNNLLGRFELSG;
- (c) a (poly)peptide comprising the amino acid sequence TKDNNLLGRFELSG;
- or
- (d) a combination of (a), (b) and/or (c).

- 30 Granzyme B is a serine protease is well known in the art and is described to be involved in the process of apoptosis/programmed cell death (Berke (1995) Cell,

81(1), 9-12; Froehlich et. al. (1998) Immunology Today, 19(1), 30-26); Metkar S et al. Cytotoxic cell-granule-mediated apoptosis: perforin delivers granzyme B serglycin complexes into target cells without plasma membrane pore information, Immunity 16 (2002), 417-428

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This enzyme promotes DNA fragmentation by cleavage of procaspases into their activated form and thereby induces programmed cell death through a Bcl-2 inhibitable pathway. Granzyme B starts to induce the process of apoptosis upon presence in the cytosol of a target cell.

10

The term "NK cells" ("natural killer cells") comprises large, granular lymphocytes expressing CD45 on the surface and exhibiting killer activity without prior stimulation. They are particularly characterised in that they do not express CD3 or T cell receptor α/β - or γ/δ and can be stimulated by interleukin-2.

15

The NK cells stimulated by the method of the invention are further characterised by the following properties:

20

- they are transient plastic-adherent after addition of IL-2 in amounts of 10 to 10,000 Units, e.g. of 100 I U, wherein IL-2 can be purchased from the firm Chiron;

25

- the adherence takes effect 3-18 hours after addition of IL-2 on newly isolated PBL (peripheral blood lymphocytes depleted by monocytes);
- the NK cells exhibit a CD16dim expression (average value of fluorescence weak);

30

- the NK cells express CD56 and CD57 as typical NK marker;
- the NK cells express CD94 (C-type lectin killer cell receptor)
- the NK cells secrete after activation with Hsp70 and cytokines IFN γ ;
- the NK cells can be stimulated by addition of Hsp70, Hsp70 fragment or Hsp70-peptide (purified protein) (growth and cytotoxic activity);
- they are not dependent on the patient's MHC type.

According to the invention, other NK-cell populations can be used, too. In this case,

however, it is a pre-requisite that they can be activated by Hsp70 or by the above-mentioned fragments or (poly)peptides. According to the invention, isolated NK cells can be used. It is furthermore possible to use cell mixtures such as peripheral mononuclear blood cells (PBMC) containing NK cells.

5

In a particularly preferred embodiment of the method of the invention peripheral blood mononuclear cells (PBMC) or a fraction thereof which contain NK cells are used as physiological cell suspensions.

Using appropriate methods, the NK cells can be obtained from the patients to be treated or from a healthy donor by taking blood. Preferably, buffy-coats (lymphocyte concentrates) containing NK cells are to be used.

Buffy-coats (lymphocyte concentrates) are taken from patients via the veins and e.g. heparin is added to prevent clotting of the cells. The buffy-coats to which heparin has been added are collected in a sterile receptacle (usually sterile plastic bags) and then centrifuged using Ficoll density centrifugation resulting in an accumulation of blood cells (=PBMC, peripheral blood mononuclear cells, e.g. lymphocytes, monocytes, granulocytes, and so on). The lymphocyte concentrate remains sterile in sterile culture bags.

The buffy-coats containing peripheral blood mononuclear cells are used in the form of a physiological cell suspension, preferably with heparin added. The heparin prevents aggregation of the cells.

Methods for the stimulation of NK cells by incubation with Hsp70 proteins of C-terminal fragments thereof have been described in WO 99 49 881. Surprisingly it has been found, that expression of granzyme B is induced or enhanced in NK cells by contacting said cells with Hsp70 protein, a fragment thereof comprising the amino acid sequence TKDNNLLGRFELSG, a (poly)peptide comprising the amino acid sequence TKDNNLLGRFELSG, or a combination of said proteins/(poly)peptides preferably in combination with IL-2. Preferably, the fragment referred to above and in connection with other (preferred) embodiments

of the invention is a carboxy-terminal (C-terminal) fragment of Hsp70.

According to the invention, the term "Hsp70 protein" relates to eukaryotic heat-shock proteins (HSPs). The expression of said HSPs can be induced by heat but
5 also by a number of other reagents such as e.g. amino acid analogues, heavy metals, ionophores or cytotoxines, wherein the factor of the increase in the expression by means of induction is at least 5, compared to the constitutive expression. The complete amino acid sequence has been published in Milner et al. (1990) Immunogenetics 32 (4), 242-251.

10 According to the invention, the term "fragment" of the Hsp70 protein also comprises (poly)peptides exhibiting an amino acid sequence from the range of amino acids 384-641 of the human Hsp70. All C-terminal (carboxy-terminal) fragments at least comprise the amino acid sequence TKDNNLLGRFELSG. Methods for the isolation of corresponding (poly)peptides are known in the art and
15 particularly described in the appended example 1. Thus, the person skilled in the art is also able to produce fragments from the above-mentioned fragment 384-641 by recombinant techniques without further ado (standard methods for this are described in Sambrook et al., "Molecular Cloning, A Laboratory Manual", 2. edition 1989, CSH Press, Cold Spring Harbor, N. Y.) and test them for the activation
20 properties wanted.

The term (poly)peptide refers to peptides as well as polypeptides (proteins). According to the conventional understanding, peptides comprise up to 30 amino acids whereas polypeptides consists of more than 30 amino acids. This convention
25 is also employed in accordance with the invention. Further, in accordance with the invention, the amino acids throughout the description are referred to by the one letter code.

In one alternative (poly)peptides comprising the amino acid sequence
30 TKDNNLLGRFELSG are (poly)peptides consisting of the recited amino acid sequence and optionally further amino acid stretches N-terminally and C-terminally

thereof derived from Hsp70, fused to further randomly chosen or naturally occurring amino acid sequences. Thus, the method of the present invention relates to the stimulation of NK cells by fusion proteins comprising the sequence of the 14-mer Hsp70-peptide.

5

A preferred embodiment of the invention relates to a method wherein the Hsp70 protein, the (C-terminal) fragment thereof, the (poly)peptide comprising the amino acid sequence TKDNNLLGRFELSG, or the combination thereof is in an uncomplexed state.

- 10 HSPs are known in the art to occur in complexes with a large number of different substrates peptides (Tamura et. al. (1997) Science, 278, 117-223). However, it has been surprisingly found that heat-shock proteins, (C-terminal) fragments thereof or derivatives derived therefrom (see above) induce immunological activities by means of activation of NK cells even if they do not form complexes with peptides.
- 15 Thus, according to the methods described in WO 99 49 881 the person skilled in the art is able stimulate NK cells using Hsp70 protein or (poly)peptide comprising the amino acid sequence TKDNNLLGRFELSG in an uncomplexed state.

According to a preferred embodiment the method of the invention is an in vivo

20 method.

A procedure envisaged would include to inject Hsp70, Hsp70 fragment or Hsp70 peptide into patients for in vivo stimulation of NK cells to produce granzyme B.

According to alternatively preferred embodiments said method is an ex vivo

25 method or an in vitro method.

This method comprises isolation of NK cells or a population of cells comprising NK cells as described herein above, wherein a physiological cell suspension containing NK cells is mixed with Hsp70 protein, the C-terminal fragment thereof or a derivative thereof or a protein/(poly)peptide comprising the amino acid sequence

30 TKDNNLLGRFELSG and incubated to induce or enhance expression of granzyme B in the NK cells.

The incubation can e.g. take place in an incubator, at physiological temperature (37°C) on a shaker (gentle shaking), at 5% CO₂ + >80% humidified atmosphere also otherwise retaining physiological conditions that allow the survival of NK cells.

5 A further preferred embodiment of the invention relates to a method further comprising reinfusion of preferably autologous and/or allogeneic NK cells with induced or enhanced granzyme B expression into a mammal.

Once the NK cells have undergone an in vitro or ex vivo treatment to induce or enhance granzyme B expression, they are re-infused into a patient. Re-infusion
10 can take effect using standard medical equipment. For example, reinfusion of NK cells or PBMC containing NK cells can be i.v., i.p., s.c., or intratumoral.

According to a further preferred embodiment of the invention said mammal is a human.

15

In another preferred embodiment of the method of the invention said contacting of the NK cells with Hsp70 protein, the (C-terminal) fragment thereof or a derivative thereof or a protein/(poly)peptide comprising the amino acid sequence TKDNNLLGRFELSG is effected for at least 12 hours. According to a further
20 preferred embodiment said contacting is effected for at least 4 days.

The present invention relates in another preferred embodiment to a method wherein said NK cells, prior to said contacting, are obtained from bone marrow cells by incubating said bone marrow cells with interleukin-15 (IL-15) and stem cell
25 factor (SCF) at concentrations of 1ng/ml – 1000 ng/ml per cytokine for at least 7 days up to 4 months.

This preferred embodiment of the invention allows for the fresh isolation of NK cells after stimulation of bone marrow cells with the named cytokines. The NK cells such obtained display the typical NK cell markers referred to herein above. Preferred
30 concentration of cytokines are in the range of 100 ng/ml for each cytokine. After stimulation with the cytokines and differentiation into NK cells which display CD94

and CD56 on their surface, contacting of these cells may proceed with Hsp70 protein or the above mentioned fragment of (poly)peptide or the combination of the above as mentioned herein before.

- 5 An alternative embodiment of the invention relates to the use of NK cells which produce (i.e. express) granzyme B after stimulation with
 - (a) Hsp70 protein;
 - (b) a (C-terminal) fragment of (a) comprising the amino acid sequence TKDNNLLGRFELSG;
 - 10 (c) a (poly)peptide comprising the amino acid sequence TKDNNLLGRFELSG; or
 - (d) a combination of (a), (b) and/or (c);
 for the preparation of a pharmaceutical composition for the treatment of tumors.
- According to the invention, pharmaceutical preparations are defined as substances
 - 15 and preparations of substances which, when used on or in the human body, are meant for healing, alleviating, preventing or recognising diseases, ailments, physical defects or pathological discomforts.
 Optionally, said pharmaceutical compositions further comprise a pharmaceutically acceptable carrier, diluent or adjuvant.
- 20 Examples of suitable pharmaceutically acceptable (tolerable) carriers are known to the person skilled in the art and comprise, for example, phosphate-buffered saline solutions, water, emulsions, such as oil/water emulsions, sterile solutions, and so on. The pharmaceutical compositions (pharmaceutical preparations) containing such carriers may be prepared according to common methods. The
 - 25 pharmaceutical compositions may be administered to the respective individuals in an appropriate dosage. Ways of administration are, for example, intravenous (i.v.), intraperitoneal (i.p.), intratumoral, subcutaneous (s.c.), intramuscular (i.m.), topic or intradermal. The dosage depends on many factors, e.g. on the patient's size, sex, weight, age as well as the type of the composition specially administered, the kind
 - 30 of administration and so on. The compositions may be administered locally or systemically. Generally, administration is carried out parenterally. Therefore, the

NK cells treated with Hsp70 protein, the C-terminal fragment thereof or a derivative thereof or a protein/(poly)peptide comprising the amino acid sequence TKDNNLLGRFELSG according to the invention are preferably injected intravenously. An injection may also be carried out directly into the tumour with an effective amount of NK cells being injected. Other known types of application are, of course, also possible. An operable number of NK cells administered includes the range of 5×10^7 to 2×10^9 NK cells, for example, as components of a leukapheresate. In such a leukapheresate, NK cells are usually present in an amount of between 5% and 20%.

Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, aqueous solutions, emulsions or suspensions, including saline such as 0.9% NaCl, phosphate buffered, X-vivo 20 etc. and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, or lactated Ringer's. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like. Furthermore, the pharmaceutical composition of the invention may comprise further agents such as interleukins or interferons depending on the intended use of the pharmaceutical composition.

According to a preferred embodiment of the invention said NK cells which are used for the preparation of a pharmaceutical composition for the treatment of tumors are stimulated by the method according to the invention.

Further, according to the invention tumors treated with said pharmaceutical composition comprise in accordance with the invention tumor cells which express

Hsp70 on the surface of their membrane.

Methods for the detection of surface expression of Hsp70 are known in the art and comprise, e.g. histological methods, flow cytometry etc..

- 5 More preferred said tumors are selected from a group consisting of stomach, gastric, colorectal, pancreas, mammary, lung, gynecological, head and neck cancer, dermatological (e.g. melanoma), neuronal tumors, leukemia and lymphoma.
- 10 The invention also relates to the use of granzyme B for the preparation of a pharmaceutical composition for the perforin-independent treatment of tumors.

A most important aspect of the present invention is mirrored by the above recited embodiment. In contrast to the speculation of the prior art, it could be shown in
15 accordance with the present invention, that granzyme B is effective in the treatment of tumors independent of the perforin-pathway. This has important applications in the strategy of treating tumors since uptake of the pharmaceutically active compound may now be devised independent of the perforin-pathway. Granzyme B could be infected intratumoral, iv, subcutan.

20

In a preferred embodiment of the use of the present invention, granzyme B is used as the only pharmaceutically active component in said pharmaceutical composition.

Again, this preferred embodiment of the invention has important implications in the
25 design of the necessary components of the pharmaceutical composition to be used in the treatment of cancers. Importantly, there is no need to include further pharmaceutically active ingredients into the pharmaceutical composition in order to effectively treat tumors and/or reduce the size of the tumors.

30 In addition, the present invention relates to a method of treating tumor comprising steps of:

- (a) contacting NK-cells with tumor cells bearing Hsp70 on their surface;
- (b) allowing granzyme B to enter the tumor cells via ion channels formed by said Hsp70 on the tumor cell surface; and
- (c) allowing said tumor cells to undergo apoptosis as a result of the enzymatic activity of granzyme B.

Ranges of tumor cells and concentrations of granzyme B as well as time ranges for the contact of granzyme B and tumor cells can be derived from the person skilled in the art by studying the above recited teachings of the invention.

In a preferred embodiment of this method of the invention, granzyme B is administered in a final concentration of 1ng/ml to 10ng/ml. It is most preferred that granzyme B is administered in a final concentration of about 6ng/ml. In this regard it is important to note that modes of administration are most preferred that deliver these concentrations of granzyme B directly to the tumor cells.

In another preferred embodiment of the use of the present invention or the method of the present invention, granzyme B is delivered/packaged in a liposome.

Encapsulation of pharmaceutically active compounds in liposomes is well established in the art.

The figures show:

Figure 1 shows the identification of granzyme B as the interacting partner for Hsp70-protein and Hsp70-peptide TKD.

Fig. 1 A: Hsp70-protein (Hsp70) and Hsp70-peptide (TKD) columns were incubated with cell lysates of the NK-cell line YT and the non-NK cell line K562. Bound proteins were eluted from the columns in 5 fractions (F1 – F5), resolved on a SDS-PAGE. Following silver stain, eluates of YT cells derived from Hsp70 and TKD columns, revealed a dominant 32 kDa protein band in fractions two (F2) and three (F3). In eluates of

K562 cells no 32 kDa protein band was detectable. The position of the 32 kDa band is indicated with an arrowhead.

Fig. 1 B: The tryptic peptides of the Coomassie-blue stained 32 kDa band of fraction 3 (F3), derived from the TKD column, correspond to human granzyme B. The probability of identification was 100% and the estimated Z-score was 1.89 corresponding to >95% confidence.

Fig. 1 C: Corresponding Western blot analysis of YT and K562 cell eluates (F3) following incubation with Hsp70-protein (Hsp70) and Hsp70-peptide (TKD) columns. The blot was autoradiographed and the localization of granzyme B was visualized by immunostaining with the granzyme B specific mAb 2C5. Eluates of YT cells (left), but not of K562 cells (right) revealed a 32 kDa granzyme B protein band.

Fig. 1 D: Intracellular flow cytometry of permeabilized YT cells (left) and K562 cells (right) using the phycoerythrin (PE)-conjugated granzyme B specific monoclonal antibody HC2-PE (solid line), as compared to an isotype-matched negative control antibody (dashed line). Only YT cells, but not K562 cells, contain cytoplasmic granzyme B.

Figure 2 shows an experiment in which apoptosis is selectively induced by isolated granzyme B (grB) in Hsp70 membrane-positive tumor cells

Fig. 2 A: Percentage of Annexin V-FITC positive and propidium iodide (PI) negative CX+ (left) and CX- (right) cells, either untreated (black bars), or following incubation either with camptothecin (4 μ g/ ml; light grey bars) or granzyme B (6 ng/ ml; dark grey bars) for 4 h, 12 h, and 24 h. The data represent the mean of three to four independent experiments \pm standard deviation; * marks values significantly different from control ($p < 0.05$).

Fig. 2 B: A representative flow cytometric analysis of Annexin V-FITC positively and propidium iodide (PI) negatively stained CX+ and CX- cells, either untreated (control), or following incubation with granzyme B (grB) for 24 h. The percentage of Annexin V-FITC positively stained

cells is given in percentage in the lower right corner of each graph.

Fig. 2 C: In parallel, either untreated (control), camptothecin (cam) or granzyme B (grB) treated CX+ and CX- cells (24 h) were stained with DAPI. Considerable nuclear DNA fragmentation was observed in CX+ and CX- cells following incubation with camptothecin (middle panel). After incubation with granzyme B only CX+ cells exhibited nuclear DNA fragmentation (lower panel, left). No signs of apoptosis was observed in CX- cells following incubation with granzyme B (lower panel, right). Scale bar represents 10 μ m.

Figure 3 shows an experiment in which kill of Hsp70 membrane-positive tumor cells is demonstrated. Apoptosis mediated by granzyme B positive NK cells is blockable by Hsp70 specific mAb.

Fig. 3 A: Light microscopy (magnification 20x) of Hsp70 membrane-positive CX+ and Hsp70 membrane-negative CX- cell colonies, either untreated (control) or following a 12 h co-incubation with Hsp70-peptide TKD stimulated NK cells (+NK). The effector to target cell ratio (E : T) was 20 : 1. Scale bar represents 200 μ m, the insert in the lower right corner of each graph shows one representative cell colony, magnification 2.5x.

Fig. 3 B: Cell kill of CX+ (left) and CX- (right) tumor target cells by naive (NK d0) and Hsp70-peptide TKD stimulated NK cells (NK d3) was quantified in 51Cr release assays. Intracellular granzyme B levels in naive NK cells (NK d0) versus TKD stimulated NK cells (NK d3) was 5-fold increased; concomitantly, lysis of CX+ cells was elevated 1.5-fold. The increased cytolytic activity of TKD stimulated NK cells (NK d3 Hsp70 mAb) against CX+ cells was completely inhibited by Hsp70 specific antibody (dashed line). Cytotoxicity was determined at E : T ratios ranging from 2 : 1 to 20 : 1; spontaneous release of each target cell was below 10%. The data represent the mean of three independent experiments \pm standard deviation.

Examples

The following examples illustrate the invention. These examples should not be construed as limiting: the examples are included for purposes of illustration and the present invention is limited only by the claims.

Example 1 Materials and Methods

Cells

The NK cell line YT was cultured at cell densities ranging from $0.1 - 0.5 \times 10^6$ cells/ml RPMI-1640 medium (Life Technologies, Eggenstein, Germany) containing 10% heat inactivated fetal calf serum (FCS, Life Technologies, Eggenstein, Germany) supplemented with 6 mM L-glutamine, and antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin; Life Technologies). Transient plastic adherent NK cells were derived from buffy coats of healthy human volunteers. Ficoll separated peripheral blood mononuclear cells (PBMC) were cultured in rIL-2 (100 IU/ml, Chiron, Frankfurt, Germany) for 12 h. Following adherence selection of monocyte depleted peripheral blood lymphocytes according to a modified method of Vujanovic (Vujanovic et. al. (1993) Cell. Immunol. 151, 133), cells were cultivated in RPMI-1640 medium supplemented with Hsp70-peptide TKD (2 µg/ml) for 3 days.

The autologous human colon carcinoma sublines CX+ and CX-, that differ with respect to their membrane expression of Hsp70, but exhibit identical MHC class I expression, and the leukemic non-NK cell line K562 were cultured in RPMI-1640 medium supplemented with 5% FCS, 6 mM L-glutamine and antibiotics.

Exponentially growing tumor cells (day 1 after cell passage) were used for granzyme B, camptothecin treatment and as targets in cytotoxicity assays.

All cell lines were screened regularly for mycoplasma contaminations by an enzyme-immunoassay detecting *M. arginini*, *M. hyorhina*, *A. laidlawii*, and *M. orale* (Roche, Mannheim, Germany). Only mycoplasma-free cell lines were used.

Affinity chromatography and immunoprecipitation

1 mg lyophilized, recombinant human Hsp70-protein (Stressgen, British Columbia,

Canada) or 2 mg Hsp70-peptide TKD (TKDNNLLGRFELSG, aa₄₅₀₋₄₆₃, Bachem, Bubendorf, Switzerland) were incubated with equilibrated AminoLink-agarose beads (Pierce, Rockford, USA) in 2 ml for 6 h, together with the reductant NaCNBH₃. Binding capacity of Hsp70-protein and Hsp70-peptide TKD was greater
 5 95%. Following removal of uncoupled material by extensive washing with Tris-buffer and quenching of non-reactive groups, cell lysates were administered to the Hsp70-protein and Hsp70-peptide TKD conjugated columns for 1 h.

After washing with 10 column volumes of 20 mM Tris buffer, bound proteins were eluted with 3 M sodium chloride in 20 mM Tris buffer, in 5 fractions. Each fraction
 10 was subjected to a 10% SDS-PAGE and blotted to PVDF membranes.

Membrane preparation

Membrane purification was performed by dounce homogenization of 50 x 10⁶ cells in hypotonic, EDTA-free buffer containing the protease inhibitor PMSF followed by sequential centrifugation at 1,000 g for 5 min and at 100,000 g, at 4°C, for 60 min.

15 The pellet containing membranes was resuspended in 2 ml 0.3 M NaCl in 50 mM Tris buffer, 0.5% NP40, pH 7.6.

Western blot analysis

Following blocking in skim-milk (0.1%) and incubation with mAb directed against granzyme B-2C5 (IgG2a, Becton Dickinson, Heidelberg, Germany), at 4°C, for 5 h,

20 Western blots were washed and incubated with a secondary mouse anti-IgG HRP Ab (Dianova, Hamburg, Germany), for 1 h. Proteins were detected using the ECL kit (Amersham Bioscience) for 5 sec.

Protein identification by peptide mass fingerprinting

The Hsp70-protein and Hsp70-peptide TKD precipitated 32 kDa protein band was
 25 cut out from Coomassie-blue stained gels, digested with trypsin, and desalted using reversed phase ZIP tips (Millipore, Eschborn, Germany). The samples were embedded in 4-hydroxy- α -cyano-cinnamic acid and the peptide masses were determined with a Perseptive Voyager DePro MALDI-TOF (Matrix Assisted Laser Desorption Ionisation – Time Of Flight) mass spectrometer in reflective mode. A
 30 peaklist was compiled with the m/z software (Proteometrics) and used for peak selection; the resulting peptide mass fingerprint was used to search the non-

redundant NCBI protein database using the Profound search engine (Proteometrics). Granzyme B was identified with 100% probability and >95% confidence.

Flow cytometry

- 5 Cells (0.5×10^6) were fixed in paraformaldehyde (1% PFA in PBS) for 10 min, and permeabilized in PBS containing BSA (0.5%), NaN_3 (0.1%), and saponin (0.1%). Then permeabilized cells were incubated either with the granzyme B-phycoerythrin conjugated monoclonal antibody HC2-PE (IgG1; Hölzel Diagnostika, Cologne, Germany) with an isotype-matched IgG1 control antibody, at 4°C for 1 h, in the
10 dark. Following washing cells were analysed on a FACSCalibur instrument (Becton Dickinson, Heidelberg, Germany).

Treatment

- Stock solutions of camptothecin (4 mg/ ml, Sigma, Munich, Germany) were diluted in DMSO and stored at 4°C in the dark. Granzyme B (6 ng/ ml, Hölzel Diagnostics, Cologne, Germany) was freshly prepared directly before usage. Exponentially
15 growing cells ($0.5 - 1.5 \times 10^6$ / ml) were incubated either with camptothecin at a final concentration of 4 µg/ ml or with purified, enzymatically active granzyme B (6 ng/ ml) (Shi et. al. (2000) Methods in Enzymology 322, 125) for 4 h, 12 h, and 24 h at 37°C. After washing in RPMI-1640 medium apoptotic cell death was determined
20 by different apoptosis assays, as described below.

Apoptosis assays

- Annexin V-FITC staining: Briefly, cells were washed twice in Hepes buffer containing 5 mM CaCl_2 and incubated with Annexin V-FITC (Roche) for 10 min at room temperature. Annexin V-FITC positively stained cells were measured on a
25 FACSCalibur flow cytometer (Becton Dickinson, Heidelberg, Germany).

- DAPI-staining: Methanol/ acetone fixed cells (0.1×10^6 cells/ 100 µl) were incubated with 0.5 µg/ µl 4,6-diamino-2-phenylindole (DAPI) in PBS/ glycerol (3 : 1), for 15 min in the dark. Following washing in PBS the cells were mounted with Fluorescent Mounting Medium (Dako, Glostrup, Denmark) and then analysed for fluorescence
30 using a Zeiss model Axioscop 2 scanning microscope (Zeiss Jena, Germany) equipped with a 40x objective and standard filters. Apoptosis was visualized with

DAPI-staining in 50 cells, each. Images were treated by multiplicative shading correction using software Axiovision (Zeiss-Vision-Jena, Germany).

Cytochrome c release: Cytochrome c release was determined using a quantitative immunoassay (DCDCO, R&D Systems, Wiesbaden, Germany). Briefly, either untreated, camptothecin (4 µg/ ml) or granzyme B (6 ng/ ml) treated CX+ and CX- cells (1.5×10^6 / ml) were washed in PBS and treated with lysis buffer for 1 h at room temperature. Following centrifugation at 1,000 g for 15 min supernatants were removed and 200 µl of a 1 : 100, 1 : 250, and a 1 : 500 dilution was used for a sandwich ELISA. Following incubation with substrate solution in the dark for 30 min the reaction was stopped. The optical density of each well was determined on an ELISA reader at 450 nm. The amount of cytochrome c was determined according to a calibration curve.

⁵¹Cr release assay and inhibition assay

NK cell mediated cytotoxicity was measured using a 12 h ⁵¹Cr radioisotope assay.

As target cells the colon carcinoma sublines CX+ and CX- were used. For blocking studies the mAb C92F3B1 and an isotype matched control antibody (IgG1) were used at a final concentration of 5 µg/ 1×10^6 cells. Following incubation of CX+ and CX- target cells with the antibodies for 30 min at 4°C, the cells were labeled with ⁵¹Cr and the cytotoxicity assay was performed as described by MacDonald (MacDonald et. al. (1974) J. Exp. Med. 140,718). The percentage of specific lysis was calculated as: [(experimental release - spontaneous release) / (maximal release - spontaneous release)] x 100.

Example 2 Granzyme B is an interacting partner of full length heat shock protein 70 (Hsp70) and of Hsp70-peptide TKD

Partner proteins were identified by affinity chromatography on immobilized human Hsp70-protein (1 mg) or a 14 amino acid peptide, coined TKD (TKDNNLLGRFELSG, aa₄₅₀₋₄₆₃, 2 mg), containing the extracellular epitope of Hsp70 that was found mediating the interaction with NK cells. This peptide was previously identified as the epitope (Reineke et. al. (1996) Immunobiol. 196, 96) of an Hsp70 specific antibody (Welch and Suhan (1986) J. Cell. Biol. 103, 2035),

which specifically detects membrane-bound Hsp70 on viable tumor cells (Multhoff et. al. (1995) *Int. J. Cancer* 61,272; Multhoff et. al. (1995) *Blood* 86,1374). A cell lysate of the NK cell line YT (Drexler et. al. (2000) *Leukemia* 14,777) was fractionated on immobilized Hsp70 or TKD peptide columns. The material bound to the columns was eluted with 3 M sodium chloride within five fractions. As a control, the non-NK cell line K562 was also fractionated on the same affinity columns. The eluted fractions were separated by SDS-PAGE (10%) and visualized by silver-staining. A dominant protein band of apparent molecular weight of 32 kDa was observed in fractions two (F2) and three (F3) of YT cell eluates derived from the Hsp70-protein (Hsp70) and the Hsp70-peptide (TKD) column (Figure 1A, YT). This band was not observed in eluates of unconjugated sepharose columns (data not shown) and in the material eluted from the affinity columns loaded with K562 cell lysates (Figure 1A, K562). Identical results were obtained with Hsp70-protein columns (data not shown). In parallel, the eluates of Hsp70-peptide TKD and Hsp70-protein derived from fraction 3 (F3) were separated by SDS-PAGE and stained with Coomassie-blue. The 32 kDa protein band derived from F3 of the Hsp70-peptide column was cut out and digested with trypsin (Figure 1B). The resulting peptides were analyzed by MALDI-TOF peptide mass fingerprinting. Sequence of the tryptic peptides exhibited 100% homology with granzyme B with an estimated Z-value was 1.89 indicating a probability of greater 95% for granzyme B (Figure 1B). The identity of the 32 kDa protein band as granzyme B was further confirmed by Western blot analysis using the granzyme B specific antibody 2C5 (IgG2a): YT cell eluates obtained from Hsp70-protein (Hsp70) and Hsp70-peptide (TKD) columns, both revealed a dominant 32 kDa granzyme B protein band (Figure 1C). Granzyme B was not detected in eluted fraction of Hsp70 or TKD affinity columns loaded with K562 cell lysates (Figure 1C). Flow cytometry using a phycoerythrin (PE)-conjugated granzyme B antibody (IgG1) again granzyme B showed positive staining for cytoplasmic granzyme B in YT cells, but not K562 cells (Figure 1D). These observations corroborate our previous results. In summary, these data indicate granzyme B is a potential partner protein of Hsp70. Granzyme B is likely to interact with the C-terminal region of Hsp70 termed TKD.

Example 3—Granzyme B induces apoptosis selectively in Hsp70 membrane-positive tumor cells

The preceding findings posed the question whether purified granzyme B can induce apoptosis of tumor cells that present Hsp70 on their cell surface. Hsp70 membrane-positive (CX+) and negative (CX-) colon carcinoma cells, that exhibit an identical MHC class I expression (Multhoff et. al. (1997) J. Immunol. 158,4341), were incubated for 4 h, 12 h, and 24 h with isolated enzymatically active granzyme B (6 ng/ ml) (Shi et. al. (2000) Methods in Enzymology 322, 125). As a positive control for apoptosis both cell types were incubated with the topoisomerase inhibitor camptothecin at a concentration of 4 µg/ ml. Apoptosis was determined by Annexin V-FITC staining measured by FACS (FACSCalibur, Becton Dickinson, Heidelberg, Germany). Apoptosis was not detected in CX+ and CX- cells incubated with camptothecin (cam) or granzyme B (grB) for 4 h incubation (Figure 2A). Both CX+ and CX- cells incubated with camptothecin underwent apoptosis after a 12 h and 24 h incubation period with camptothecin. As shown in Figure 2A, after 12 h the amount of Annexin V-FITC positively stained CX+ cells increased from 16.6% to 28.0% (1.7-fold) and after 24 h from 18% to 39% (2.1-fold). In CX- cells the amount of Annexin V-FITC positively stained cells increased similarly from 12.1% to 19.8% (1.6-fold) after 12 h, and from 11.9% to 25.1% (2.1-fold) after 24 h. In contrast, apoptosis was observed selectively in Hsp70 membrane-positive CX+ cells, incubated with granzyme B for 12 h and 24 h, with an increase of 16.6% to 20.9% (1.2-fold), and of 18% to 30.2% (1.7-fold), respectively. In CX- cells neither a 12 h nor a 24 h treatment with granzyme B induces apoptosis. A comparative Annexin V-FITC staining pattern of CX+ and CX- cells treated with granzyme B for 24 h, is illustrated in Figure 2B. Compared to untreated control cells (18%), the amount of Annexin V-FITC positively and propidium iodide (PI) negatively stained CX+ cells increased 1.7-fold (30%) following treatment with granzyme B. However, the amount of apoptotic CX- cells remained unaltered before and after identical treatment with granzyme B. In addition to CX+ cells, granzyme B induces apoptosis also in the Hsp70 membrane-positive leukemic cell line K562 (data not

shown). The amount of Annexin V-FITC positively stained K562 cells increased from 8.7% up to 16% (1.8-fold).

Consistent with the results from Annexin V-FITC staining pattern both cell types exhibited nuclear fragmentation, a typical sign of apoptosis, as detected by DAPI-staining of nuclear DNA following treatment with camptothecin (4 $\mu\text{g}/\text{ml}$) for 24 h (Figure 2C, cam). However, DNA fragmentation was detected only in Hsp70 membrane-positive CX+ cells after 24 h of granzyme B treatment (6 ng/ ml), whereas no DNA fragmentation was observed in Hsp70 membrane-negative CX-cells (Figure 2C, grB).

As an additional test for apoptotic cell death, cytochrome c release was measured following incubation of CX+ and CX- cells with granzyme B for 24 h. As summarized in Table I, following incubation with granzyme B (6 ng/ ml) for 24 h, cytochrome c concentration was elevated from 0.382 mg/ ml to 0.690 mg/ ml (1.8-fold) in CX+ cells. However, no increase in cytochrome c was observed in CX- cells following treatment with granzyme B (0.452 mg/ ml versus 0.425 mg/ ml). In contrast, an incubation with camptothecin (4 $\mu\text{g}/\text{ml}$) for 24 h, results in a comparable 1.5-fold increase in cytochrome c concentrations in both cell types. These results indicate that isolated granzyme B induces apoptotic cell death selectively in tumor cells presenting Hsp70 on their cell surface. It was propose that the trigger of apoptosis by granzyme B is mediated via the extracellular exposed Hsp70 epitope TKD.

Table I

Quantitative determination of human cytochrome c in CX+ and CX- tumor cells either untreated (control), or following incubation with camptothecin (4 $\mu\text{g}/\text{ml}$) or granzyme B (6 ng/ ml) for 24 h. The data represent the mean of 2 independent experiments; * marks values significantly different from control ($p < 0.05$).

cells	cytochrome c (mg/ ml)		
	fold increase		
	control	camptothecin	granzyme B
CX+	0.382 ± 0.02	0.555 ± 0.04*	0.690 ± 0.08*
	1.0	1.5	1.8
CX-	0.452 ± 0.02	0.672 ± 0.02*	0.425 ± 0.075
	1.0	1.5	0.9

Example 4 Stimulation of NK cells with Hsp70-peptide TKD induces the production of granzyme B and increases kill of Hsp70 membrane-positive tumor target cells

The physiological role of our findings was tested in functional assays using naive and Hsp70 stimulated human NK cells. Previously, it was shown that incubation of NK cells with Hsp70-protein at concentrations between 10 and 50 µg/ ml or with equivalent Hsp70-peptide concentrations (0.2 – 2.0 µg/ ml) resulted in increased cytolytic activity of NK cells against Hsp70 membrane-positive tumor target cells. Concomitantly, the expression of the killer cell activating C-type lectin receptor CD94 was unregulated (Multhoff et. al. (1999) Exp. Hematology 27,1627; Gross et. al. (2002) submitted). Although Hsp70 acts as a tumor-selective recognition structure for NK cells, as determined by antibody blocking studies (Multhoff et. al. (1997) J. Immunol. 158,4341; Multhoff et. al. (1995) Blood 86,1374), the NK-cytotoxic mechanism remains unclear. To elucidate the possible mechanism, NK cells were incubated with Hsp70-peptide TKD (2 µg/ ml) for 3 days. A significant elevated intracellular granzyme B expression, as determined in 3 independent experiments, was observed. In contrast, granzyme B expression was not increased in CD3 positive T cells treated with Hsp70-peptide TKD. Light microscopical analysis of Hsp70-peptide activated NK cells co-incubated with Hsp70 membrane-positive CX+ and Hsp70 membrane-negative CX- cells is illustrated in Figure 3A. Hsp70 membrane-positive CX+ and Hsp70 membrane-negative CX- tumor cells (0.1 x10⁶ cells/ ml) were cultured in duplicates in 24 well plates for 2 days. The

proliferation rate of both tumor cell lines was comparable, as determined by identical cell counts (0.3×10^6 cells/ ml). CX+ and CX- tumor cells in the upper panel were cultured in the absence of NK cells; tumor cells in the lower panel were co-cultured for 12 h with NK cells that had been stimulated with Hsp70-peptide TKD (2 μ g/ ml, 3 days). Nearly 100% of the CX+ cell colonies were found in clusters with NK cells and viability of CX+ tumor cells appears to be reduced. In contrast, CX- tumor cells and NK cells were not found in clusters; Hsp70 membrane-negative CX- tumor cells did not attract NK cells. Cell viability of CX-tumor cells following contact with NK cells appears to be less affected, as compared to that of CX+ tumor cells. The inserts in the lower right corner of each graph illustrates a 2.5x magnification of one representative cell colony.

Cell kill of CX+ and CX- tumor cells following contact with freshly isolated, unstimulated (NK d0) or Hsp70-peptide TKD stimulated NK cells (NK d3) was quantitated in a 12 h ^{51}Cr release assay (Figure 3B). Consistently with what was observed in light microscopy (Figure 3A), the cytolytic activity of TKD stimulated NK cells (NK d3) against CX+ target cells (left) was significantly enhanced as compared to CX- target cells (right). Concomitant with the increased granzyme B levels following stimulation with Hsp70-peptide TKD for 3 days, the cytolytic response against Hsp70 membrane-positive CX+ cells, but not against Hsp70 membrane-negative CX- cells was significantly elevated; 1.5-fold at E : T ratios of 5 : 1 to 20 : 1. Since CX+ and CX- tumor cells differ only with respect to their Hsp70 membrane expression but exhibit an identical MHC class I expression pattern the inhibitory effect mediated by killer cell inhibitory receptors (KIR) could be excluded. The increased cytolytic activity against Hsp70 membrane-positive CX+ tumor cells (left), but not against Hsp70 membrane-negative CX- tumor cells (right), could be completely inhibited by pre-incubation of the target cells with the Hsp70 specific monoclonal antibody that is known to detect membrane-bound Hsp70-peptide TKD on viable tumor cells (Multhoff et. al. (1995) *Int. J. Cancer* 61,272). Therefore, it was hypothesized that interaction of granzyme B with membrane-bound Hsp70-peptide TKD is key for its uptake into tumor cells and for the induction of apoptosis. When NK cells were removed by washing with PBS and the tumor cells were

stained with DAPI. Hsp70 membrane-positive CX+ cells showed DNA fragmentation, whereas Hsp70 membrane-negative CX- cells not following co-incubation with NK cells. Identical results were obtained with Annexin V-FITC staining (data not shown). These observations strongly suggests that TKD
5 activated NK cells kill Hsp70 membrane-positive CX+ cells by induction of apoptosis, which also have elevated levels of granzyme B.

Claims

1. A method of inducing or enhancing the expression of granzyme B in natural
5 killer (NK) cells comprising contacting NK cells with
 - (a) Hsp70 protein;
 - (b) a (C-terminal) fragment of (a) comprising the amino acid sequence TKDNNLLGRFELSG;
 - (c) a (poly)peptide comprising the amino acid sequence
10 TKDNNLLGRFELSG; or
 - (d) a combination of (a), (b) and/or (c).
2. The method of claim 1, wherein the Hsp70 protein, the (C-terminal) fragment
15 thereof, the (poly)peptide comprising the amino acid sequence TKDNNLLGRFELSG, or the combination thereof is in an uncomplexed state.
3. The method of claim 1 or 2, which is an in vivo method.
4. The method of claim 1 or 2, which is an ex vivo method.
5. The method of claim 1 or 2, which is an in vitro method.
6. The method of claim 4 further comprising reinfusion of NK cells with induced
20 or enhanced granzyme B expression into a mammal.
7. The method of claim 6, wherein the reinfused NK cells are autologous and/or allogeneic NK cells.
8. The method of claim 6 or 7, wherein said mammal is a human.
9. The method of any one of claims 1 to 8 wherein said contacting is effected
25 for at least 12 hours.

- ~~10. The method of claim 9, wherein said contacting is effected for at least 4 weeks.~~
- 5 11. The method of any one of claim 1 to 10 wherein said NK cells are prior to said contacting, obtained from bone marrow by incubating said bone marrow cells with interleukin-15 (IL-15) and stem cell factor (SCF) at concentrations of 1ng/ml – 1000 ng/ml per cytokine for at least 7 days up to 4 months.
- 10 12. Use of NK cells which produce granzyme B after stimulation with
(a) Hsp70 protein;
(b) a (C-terminal) fragment of (a) comprising the amino acid sequence TKDNNLLGRFELSG;
(c) a (poly)peptide comprising the amino acid sequence TKDNNLLGRFELSG; or
15 (d) a combination of (a), (b) and/or (c);
for the preparation of a pharmaceutical composition for the treatment of tumors.
13. The use according to claim 12 wherein the NK cells are stimulated by a method according to any of claims 1 to 11.
- 20 14. The use of claim 12 or 13 wherein said tumors comprise tumor cells which express Hsp70 on the surface of their membrane.
15. The use of claim 14 wherein said tumors are selected from a group consisting of stomach, gastric, colorectal, lung, pancreas, mammary, gynecological, head and neck tumors, dermatological tumors (e.g.
25 melanoma), neuronal tumors, leukemia and lymphoma.
16. Use of granzyme B for the preparation of pharmaceutical composition for the perforin-independent treatment of tumors.

17. The use of claim 16 wherein granzyme B is used as the only pharmaceutically active compound in said pharmaceutical composition.

18. A method treating tumor comprising of:

(a) contacting NK-cells with tumor cells bearing Hsp70 on their surface;

5 (b) allowing granzyme B to enter the tumor cells via ion channels formed by said Hsp70 on the tumor cell surface; and

(c) allowing said tumor cells to undergo apoptosis as a result of the enzymatic activity of granzyme B.

10 19. The method of claim 18, wherein granzyme B is administered in a final concentration of 1ng/ml to 10 ng/ml.

20. The method of claim 19 wherein granzyme B is administered in a final concentration of about 6 ng/ml.

15

21. The use of claim 16 or 17 or the method of any one of claims 18 to 20 wherein granzyme B is packaged in liposomes.

Abstract

5 The present invention relates to a method of inducing or enhancing the expression of granzyme B in natural killer (NK) cells. The present invention relates also to a use of said NK cells for the preparation of a pharmaceutical composition for the treatment of tumors.

Fig. 1 A

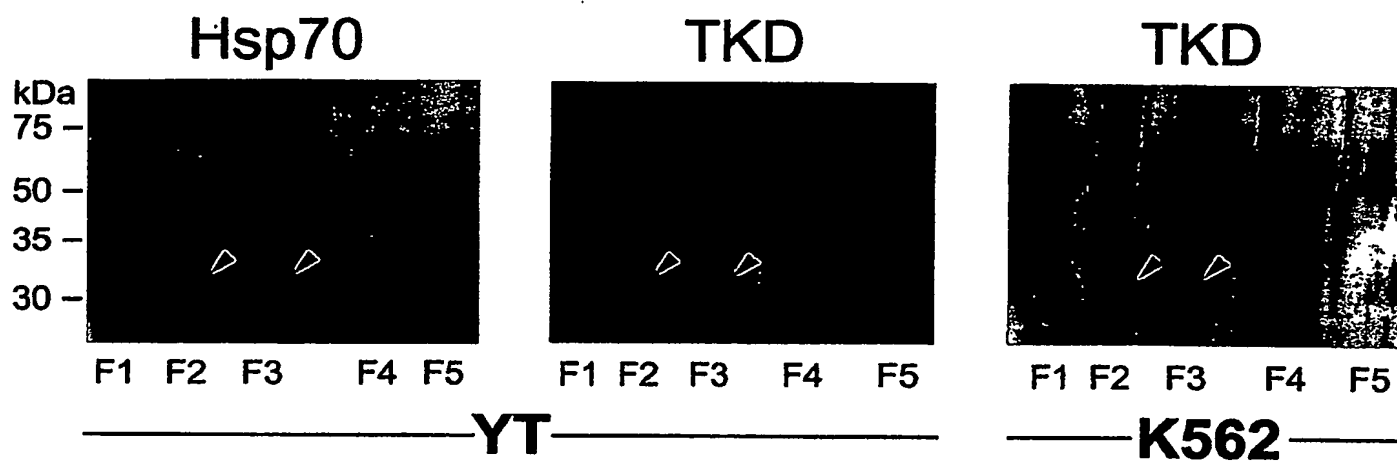


Fig. 1 B**Identification of human granzyme B by MALDI-TOF analysis**

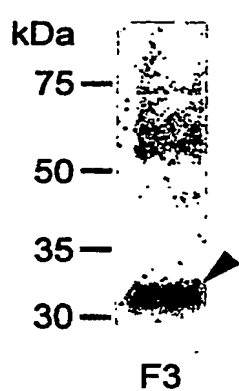
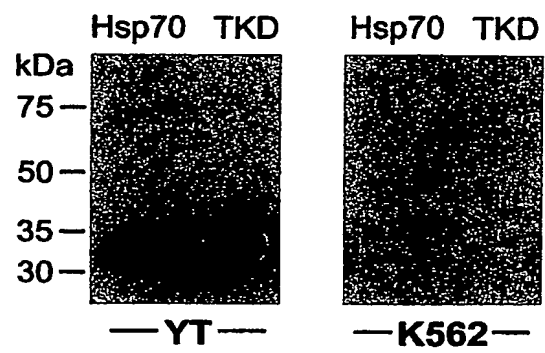
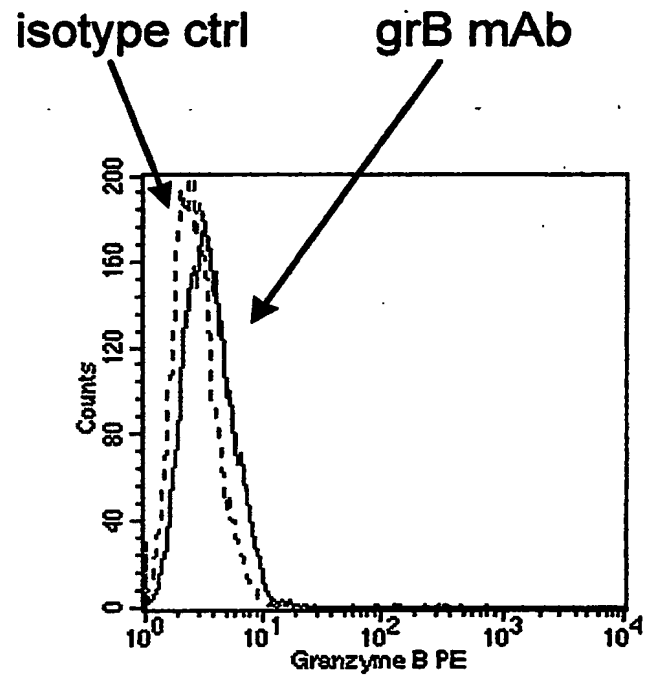
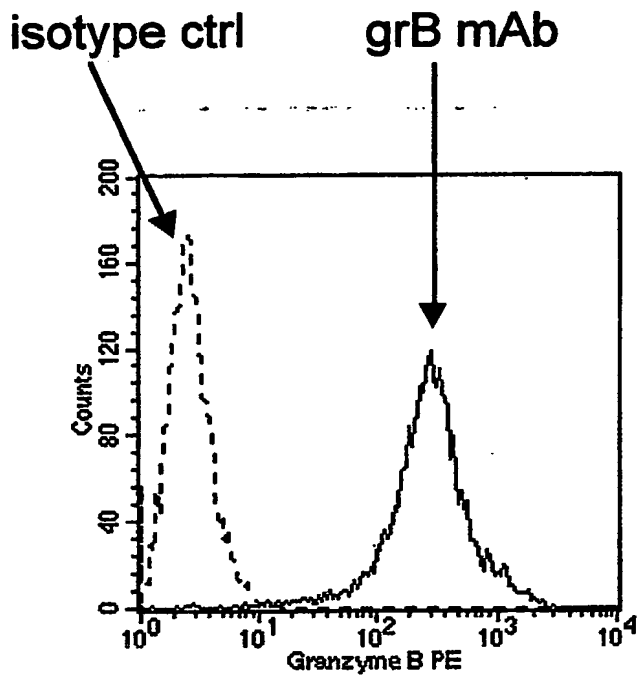
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	893.369	893.391	145	151	MTVQEDR
	1048.572	1048.566	192	201	VAGIVSYGR
	1055.549	1077.556	136	144	HSHTLQEVKV
	1288.702	1288.703	73	83	RPIHPAYNPK
	1442.722	1442.740	61	72	EQEPTQQFIPVK

Fig. 1 C

YT**K562****Fig. 1 D**

CX+

5/9

CX-

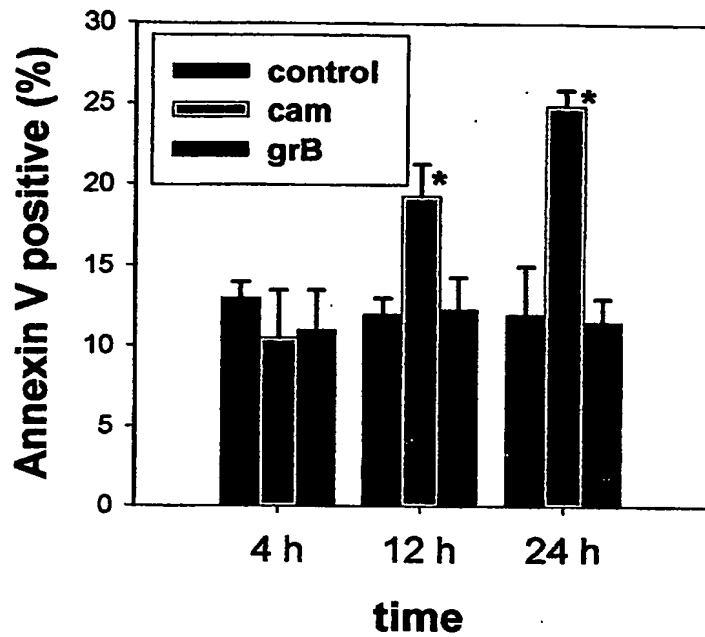
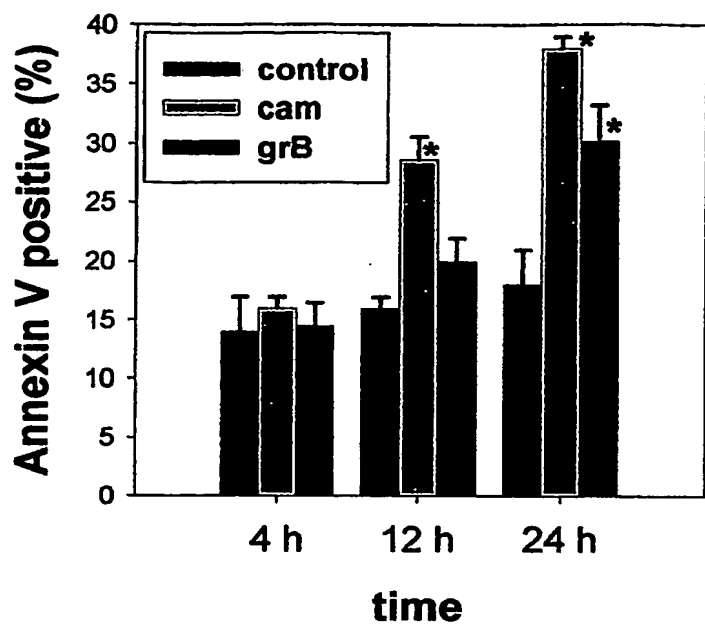
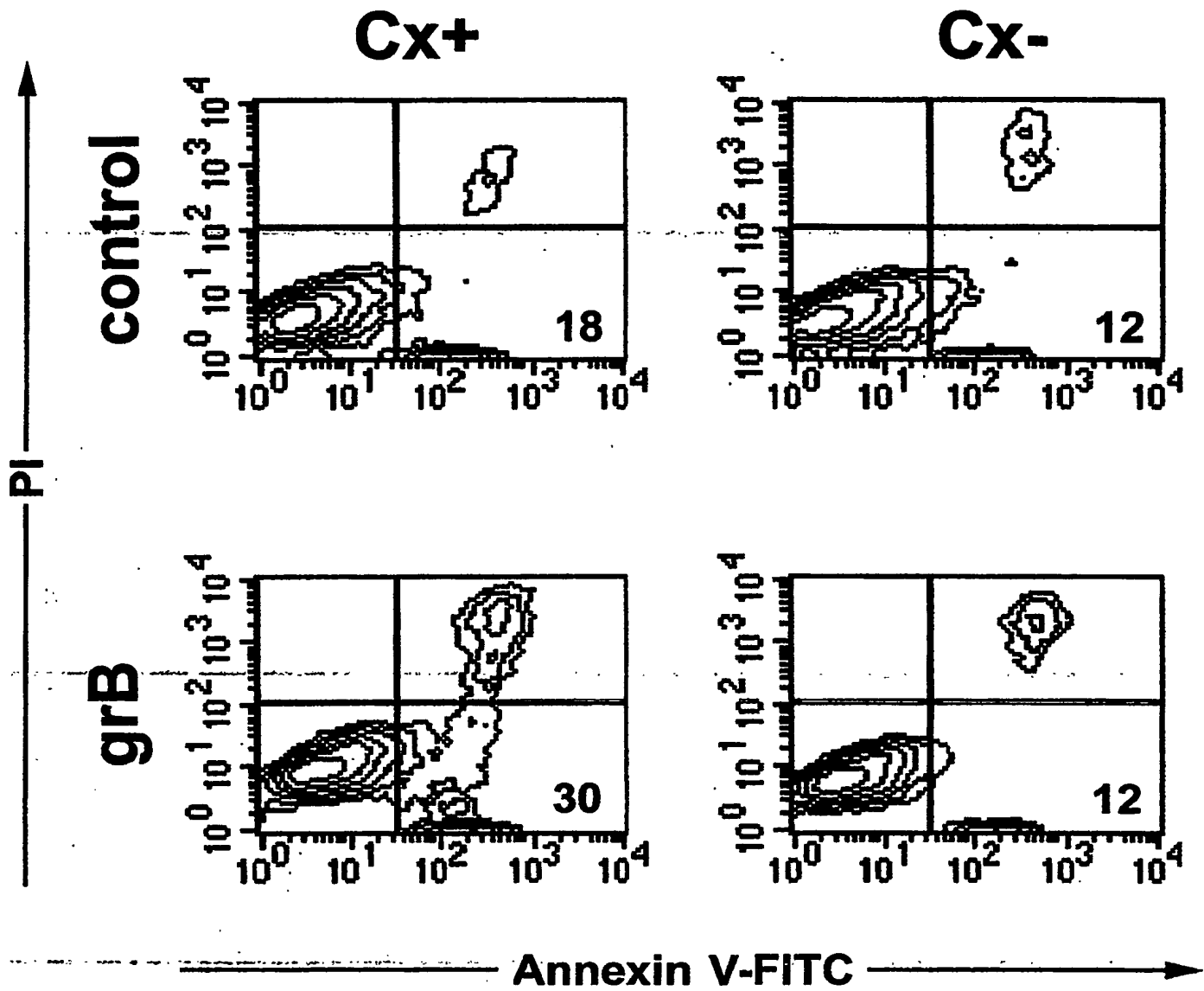


Fig. 2 A

Fig. 2 B



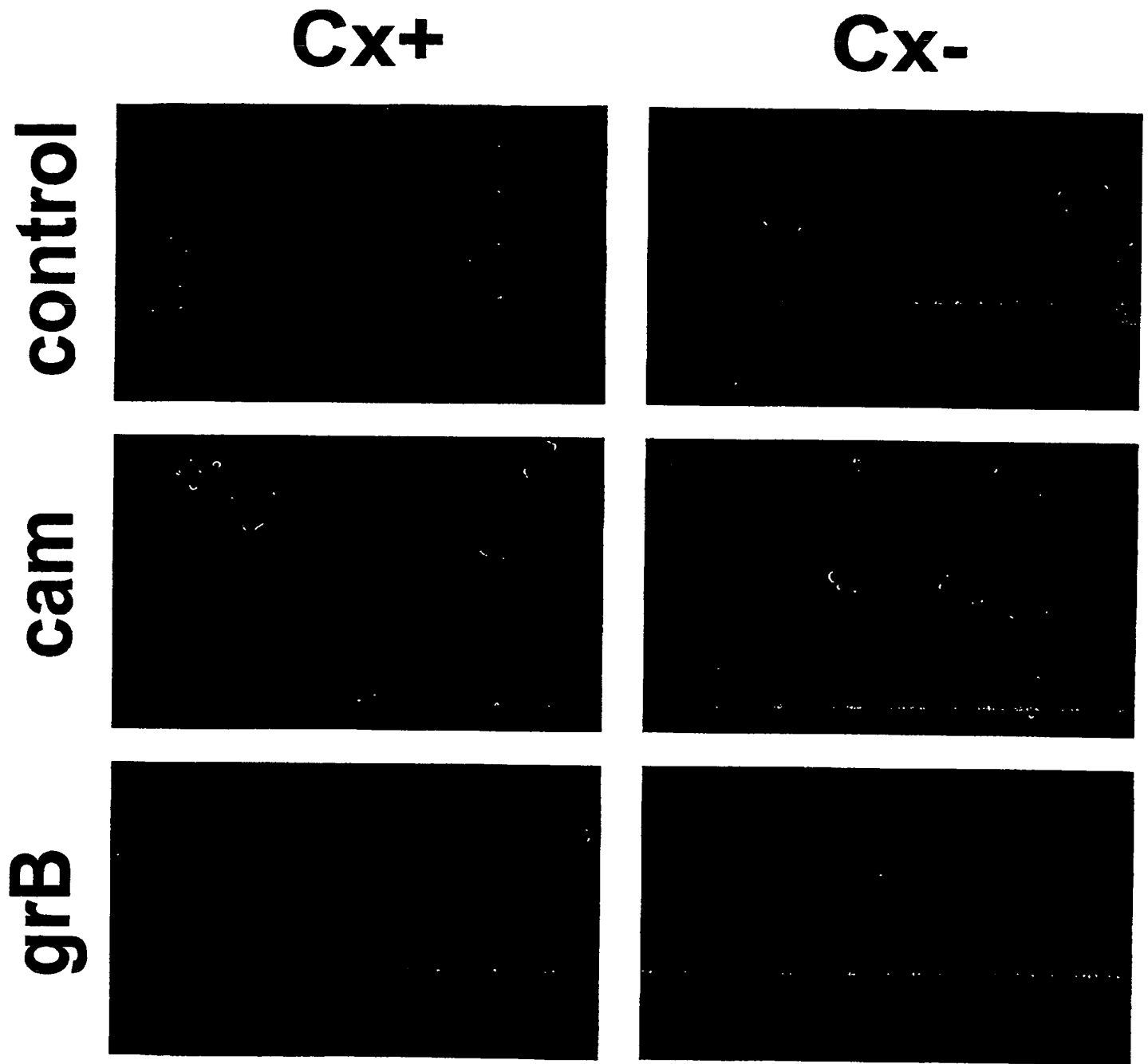


Fig. 2 C

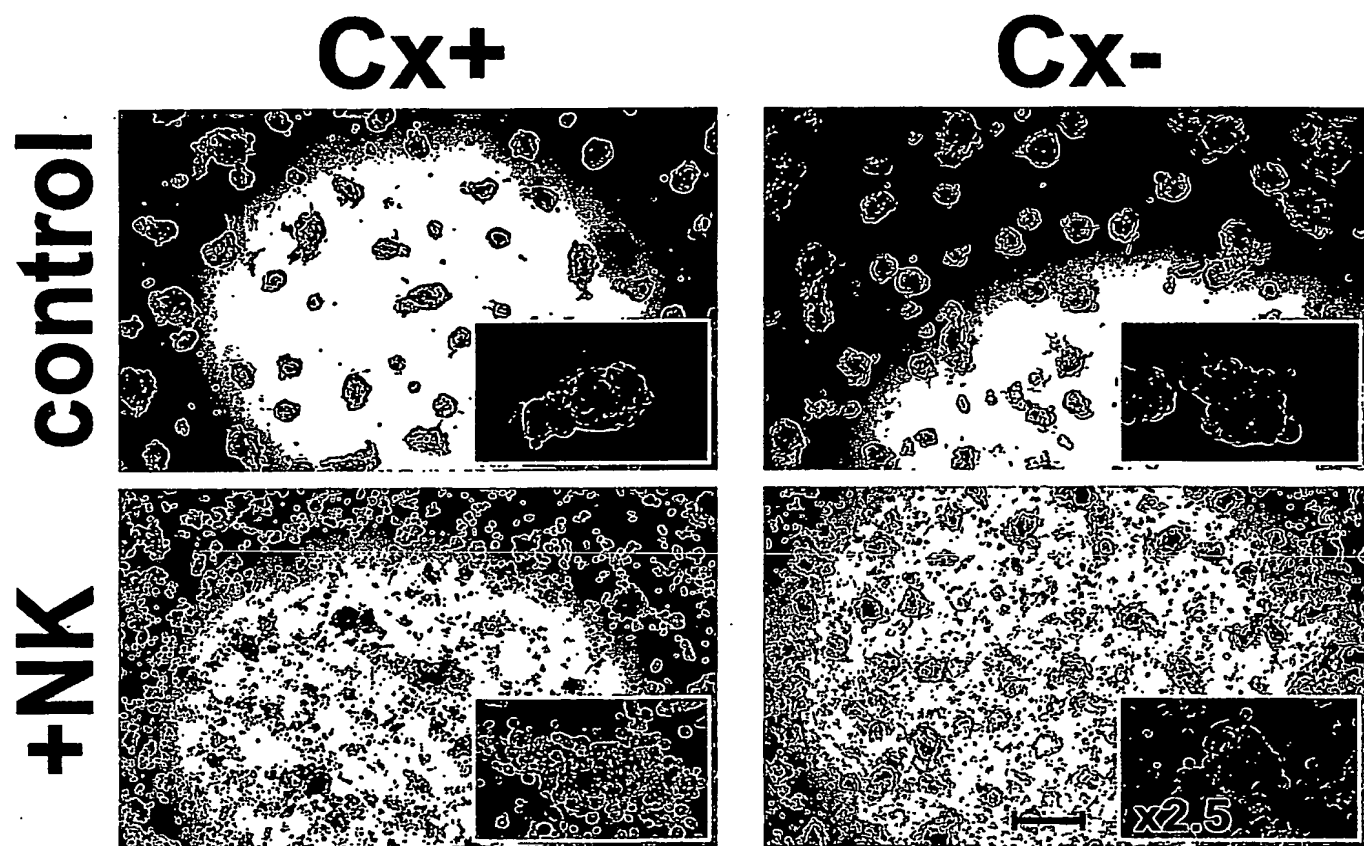
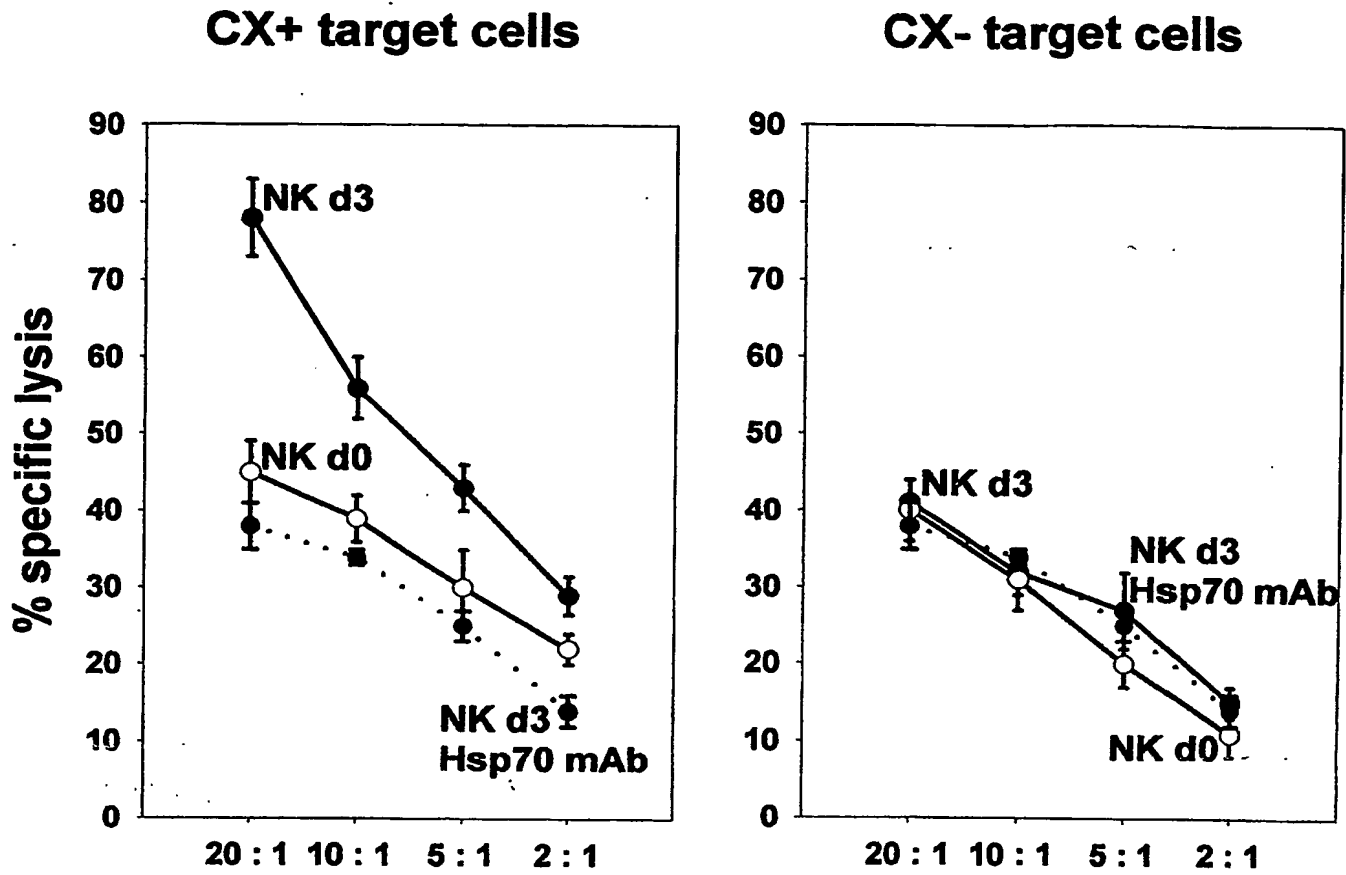


Fig. 3 A

**Fig. 3 B**

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